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Research Article

Protein Profile and Plasmid Content of *Lactococcus lactis* subsp. *lactis* LL52 and *Lactococcus lactis* subsp. *cremoris* LC79 Strains under Several Stress Conditions

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Abstract: Differences in the protein and plasmid content of 2 *Lactococcus lactis* strains, *L. lactis* subsp. *lactis* LL52 and *L. lactis* subsp. *cremoris* LC79, under the stresses of high and low temperature, osmotic shock, and low pH were determined. We identified 3 new proteins with molecular masses of 16.0, 29.4, and 45.0 kDa as high temperature stress response specific in strain LL52. High temperature stress did not cause any changes in the protein content of strain LC79. Proteins that were specific for salt stress and low pH stress responses ranged between 16.0 and 40.5 kDa, and 24.8 and 107.5 kDa, respectively, in both strains. No proteins were related to low temperature stress in LL52 and LC79 strains. Plasmid analysis indicated that there was no relationship between stress responses and plasmids in these bacteria.

Key Words: *Lactococcus lactis*, stress, protein, plasmid

Bazı Stres Koşulları Altında *Lactococcus lactis* subsp. *lactis* LL52 ve *Lactococcus lactis* subsp. *cremoris* LC79 Suşlarının Protein Profilleri ve Plazmid İçerikleri

Özet: İki *Lactococcus lactis* suşu olan *L. lactis* subsp. *lactis* LL52 ve *L. lactis* subsp. *cremoris* LC79'un yüksek ve düşük sıcaklık, ozmotik şok ve düşük pH stresleri altında protein profilleri ve plazmid içeriklerindeki değişimler belirlendi. LL52 suşunda yüksek sıcaklık stresine yanıt olarak 16,0, 29,4 ve 45,0 kDa moleküler ağırlıkta üç farklı spesifik protein tespit edildi. LC79 suşunda yüksek sıcaklık stresi protein içeriğinde herhangi bir değişime neden olmadı. Her iki suşta tuz ve düşük pH stres yanıtlarına spesifik proteinler, sırasıyla 16,0-40,5 kDa ve 24,8-107,5 kDa aralığında değişim gösterdi. Düşük sıcaklık stresiyle ilişkili olarak LL52 ve LC79 suşlarında hiçbir protein bulunamadı. Plazmid analizleri, stres yanıtları ile bakterilerideki plazmidler arasında bir bağlantının bulunmadığını gösterdi.

Anahtar Sözcükler: *Lactococcus lactis*, stres, protein, plazmid

Introduction

Lactococcus lactis is a commercially important group of bacteria because of its extensive use in the production of dairy foods and nisin (1). These bacteria are exposed to various harsh conditions imposed by industrial processes (2). The end of fermentation imposes a hostile environment on lactococci in dairy products, which involves low pH, the absence of nutrients, unfavorable osmotic conditions, and high or low temperatures (3-6). Optimal conditions for growth are rare and require specific adaptations, which can then also act as stress conditions (7). From an industrial point of view it is important to select strains that perform well in fermentation and are resistant to the adverse conditions that occur during the fermentation process (8). In addition, such strains should survive the storage and handling procedures that dairy products undergo during industrial processing (9,10). Therefore, further investigations of the stress responses of *L. lactis* under different conditions are required (3,6). Although the

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stress response has been studied extensively in some organisms, only limited work on osmotic stress, low pH stress, and freezing stress has been reported for *L. lactis* (1,2,11).

The focus of this study was the investigation of plasmid and protein content under high and low temperature, salt, and low pH stress conditions in 2 *L. lactis* strains, *L. lactis* subsp. *lactis* LL52 and *L. lactis* subsp. *cremoris* LC79.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions

L. lactis strains used in this study were obtained from the Ankara University Biology Department Culture Collection. Lactococcal cultures were grown at 30 °C in M17 broth (12), with the pH adjusted to 7.4. Glucose replaced lactose in M17 medium (M17glu) when necessary. Bacterial stocks were stored until use in M17 broth containing 40% glycerol at –18 °C.

Bacterial Survival under Stress Conditions

Bacterial strains were grown in 5 ml of M17glu medium at 30 °C until stationary phase (approximately 8 h, OD_{600} of 1.2-1.6). Following incubation (time zero), a sample was collected and colony forming units (CFU) were counted. *L. lactis* cultures were then dispensed into 4 tubes. After centrifugation, cell pellets were resuspended with 10 ml of M17glu. To measure tolerance to stresses, cells were challenge treated with the following: (i) 30 °C at pH 2.5 (*L. lactis* subsp. *lactis* LL52) and at pH 3.0 (*L. lactis* subsp. *cremoris* LC79); (ii) 0.1% bile salt (*L. lactis* subsp. *lactis* LL52) and 0.04% bile salt (*L. lactis* subsp. *cremoris* LC79); (iii) heat (45 °C) (both strains); (iv) cold (cultures were directly frozen at –18 °C) (both strains). Samples were removed at specified time intervals and diluted in 0.9% NaCl and then 0.1 ml of each sample was plated onto M17glu medium to measure the survival rate. Survival was determined as the ratio of CFU after challenge treatment to CFU at time zero (1,3).

Protein Extraction and Analysis

Total cellular proteins from equal numbers of cells were prepared according to Boutibonnes et al. (13). Harvested cells were washed twice in sterile distilled water, resuspended in 1.5 ml of distilled water (A_{600} nm = 2), and centrifuged. Proteins were extracted from the final pellets with 0.5 ml of 0.001 mol l^{-1} EDTA, 0.01 mol l^{-1} Tris-HCl, 0.01 mol l^{-1} NaCl, and 2% SDS, pH 8.0, at 100 °C for 5 min. Proteins were subjected to 12.5% sodium dodesil sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed on a Pharmacia PhastSystem (Pharmacia, Uppsala, Sweden) and gels were stained with Coomassie blue (14).

Plasmid DNA Isolation and Agarose Gel Electrophoresis

Plasmid DNA was extracted from cells according to the lysis procedure described by Anderson and McKay (15). Pure plasmid samples were prepared by CsCl-gradient ultracentrifugation (16). Samples of plasmid DNA were analyzed by agarose (0.7%) gel electrophoresis in TBE buffer (0.089 M Tris base, 0.089 M boric acid, 0.002 M Na-EDTA, pH 8.3) at 100 V for 3.5 h.

Results

It is well established that bacteria can differ in their stress responses to various conditions and that they can either produce new proteins or modify some existing proteins to adapt to such conditions. In the present study we investigated the stress responses of 2 *L. lactis* strains, *L. lactis* subsp. *lactis* LL52 and *L. lactis* subsp. *cremoris* LC79, which were selected due to their industrial importance. The strains were exposed to high and low temperature, high salt, and low pH. The obtained results indicated that both strains were tolerant to high and low temperature, and low pH stresses; their survival rates varied between 51.3% and 90.6% (Table). In contrast, during bile salt treatment the cell survival rate of both strains dropped drastically within the first 30 min and then remained constant.

Changes in the protein and plasmid content of the 2 *L. lactis* strains were determined after 30 and 90 min of exposure to different stress conditions (Figures 1-4). After 30 min of exposure to 45 °C, 3 weak bands, 16.0, 29.4, and 40.5 kDa, were present in *L. lactis* subsp. *lactis* LL52, but not after 90 min (Figure 1). In contrast, the high temperature challenge did not seem to have any effect on the stability of the 3 plasmids (7.5, 9.0, and 30.4 kb) of the studied strain (Figure 2). During low pH treatment, the intensity of the bands of 59.3, 40.5, and 75.8 kDa proteins decreased, whereas proteins of 83.4, 93.0, 102.5, and 107.5 kDa were not detected at all

Table. Stress tolerance of *L. lactis* cells.

Strains and Stress Conditions	% Survival	
	30 min	90 min
LL52[1]		
Control	99.6	99.4
Bile salts (0.1%)	17.4	17.3
Acid (pH 2.5)	51.6	51.4
Heat (45 °C)	63.5	62.8
Cold (–18 °C)	90.6	87.1
LC79[2]		
Control	99.8	99.4
Bile salts (0.04%)	13.1	13.7
Acid (pH 3.0)	53.5	51.3
Heat (45 °C)	58.3	56.4
Cold (–18 °C)	88.6	90.2

[1] *L. lactis* subsp. *lactis* LL52.
[2] *L. lactis* subsp. *cremoris* LC79.

(Figure 1). Similar to the high temperature treatment, plasmid content was stable during the low pH procedure (Figure 2).

We used bile salt to determine the responses to osmotic shock. Differences observed in proteins were dependent on the duration of stress exposure. Compared to the wild-type, while there was a new 40.5 kDa protein produced in the first 30 min, there were 2 additional proteins of 16.0 and 29.4 kDa after 90 min (Figure 1). No plasmid loss was recorded after 30 min of osmotic stress; however, 7.5 and 9.0 kb plasmids were missing after 90 min (Figure 2). The last stress condition, cold stress, had no effect on either the plasmid or protein content in LL52 cells during 30 and 90 min of exposure (Figures 1 and 2).

In *L. lactis* subsp. *cremoris* LC79, only the 42.4 kDa protein production was inhibited after 30 min of the high temperature treatment (Figure 3). No changes were detected in the plasmid content of the cells subjected to high temperature after 30 min; however, 36.6, 32.5, 7.1, and 5.2 kb plasmids were lost after 90 min (Figure 4). In LC79 cells under the low pH stress condition, although the stability of the plasmids was not affected, 24.8, 31.0, 33.8, and 42.4 kDa proteins could not be detected after 30 min (Figures 3 and 4). Furthermore, after 30 min of osmotic stress the 31.0 and 33.8 kDa proteins were not detected, while after 90 min these proteins were present (Figure 3). During osmotic stress the 7.1 kb plasmid could not be detected in the cells (Figure 4). Finally, under low temperature, along with the other 2 strains, the total plasmid and protein content of treated LC79 cells was similar to that of the wild-type (Figures 3 and 4).

Discussion

Bacteria either produce or modify proteins (or both) to improve their adaptation to stress conditions, such as low and high temperature, high osmotic pressure, and low pH, which might occur, especially during the fermentation process (17,18). At least 14 different proteins produced by different species of bacteria have been examined. These include cold shock protein and CspA, which modifies other proteins. (7). Similarly, under heat stress, along with DnaK, which is the primary chaperone that maintains bacterial adaptation (DnaK is a 70-kDa complex of DnaJ and GrpE), many proteins are either produced or modified (19,20). During adaptation to these conditions cells also increase the concentration of particular metabolites, such as glycine, betaine, proline, and fatty acids (7,11,21). All these biological processes require the participation of different enzyme systems at different stages. Moreover, the induction of chaperon proteins, which are responsible for the correct folding of partially or improperly folded proteins, is well maintained in all of the stress conditions mentioned above (17,18,22).

The present study found that *L. lactis* subsp. *lactis* LL52 and *L. lactis* subsp. *cremoris* LC79 strains had 4 basic differences in terms of protein content under the stress conditions used: 1. Production of some new proteins not present in the wild-type strain; 2. Inhibition of production of some proteins that are produced by the wild-type strain; 3. Increase in the level of expression of some proteins; 4. Decrease in the level of expression of some proteins that are present in the wild-type strain. All 4 of the above differences are directly associated with the response of *L. lactis* strains to different stress conditions. The production of novel proteins or the increased production of already existing proteins, which are only produced under stress conditions, is responsible for stress responses. The decrease in production or the

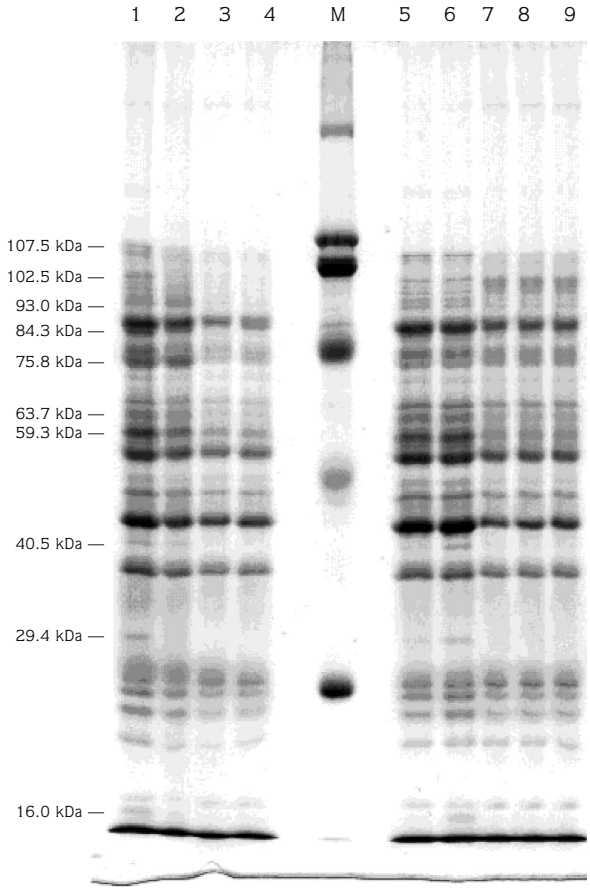

Figure 1. Protein profiles of *L. lactis* subsp. *lactis* LL52 under normal and stress conditions: 1. High temperature, 30 min; 2. High temperature, 90 min; 3. Low pH, 30 min; 4. Low pH, 90 min; 5. Osmotic stress, 30 min; 6. Osmotic stress, 90 min; 7. Low temperature, 30 min; 8. Low temperature, 90 min; 9. Wild-type, M: protein marker (205, 116, 97, 4, 66, 45, and 29 kDa).

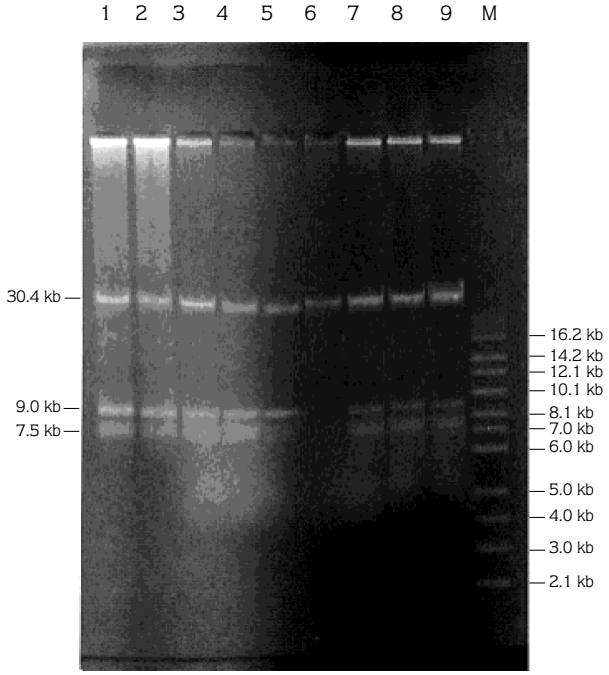

Figure 2. Plasmid profiles of *L. lactis* subsp. *lactis* LL52 under normal and stress conditions: 1. High temperature, 30 min; 2. High temperature, 90 min; 3. Low pH, 30 min; 4. Low pH, 90 min; 5. Osmotic stress, 30 min; 6. Osmotic stress, 90 min; 7. Low temperature, 30 min; 8. Low temperature, 90 min; 9. Wild-type, M: plasmid marker.

inhibition of production of certain proteins is most probably the result of high levels of protein modification or gene regulation, caused by a decrease in metabolic activity. To confirm such speculations, it is important to examine the proteins at the molecular and biochemical levels. Specifically, in the LL52 strain, production of 3 new proteins with molecular masses of 16.0, 29.4, and 40.5 kDa, under both high temperature and osmotic stress, confirmed other literature reports of similar proteins being produced under these 2 stress conditions (1,23). Decreased production of the 42.4 kDa protein under high temperature and low pH in the LC79 strain was also inconsistent with conventional literature findings (7,18,24,25); thus, it is even more crucial to identify these proteins at the molecular level.

On the other hand, the ability of the tested strains, which originated in Turkey, to adapt quickly to the stress conditions (30 or 90 min after treatment), which also occur frequently in industrial production, indicates that these bacteria are appropriate for starter culture strain development programs.

Under all stress conditions, the common behavior of bacteria is the confinement of metabolic activity, and the most conventional way to accomplish this is to eliminate the existing plasmids (26,27). In Turkey-originated strains of *L. lactis* no linear relationship between the proteins, either induced or inhibited, and the plasmid content of these strains was observed. In addition, only limited plasmid loss took place. In *L. lactis* strains many

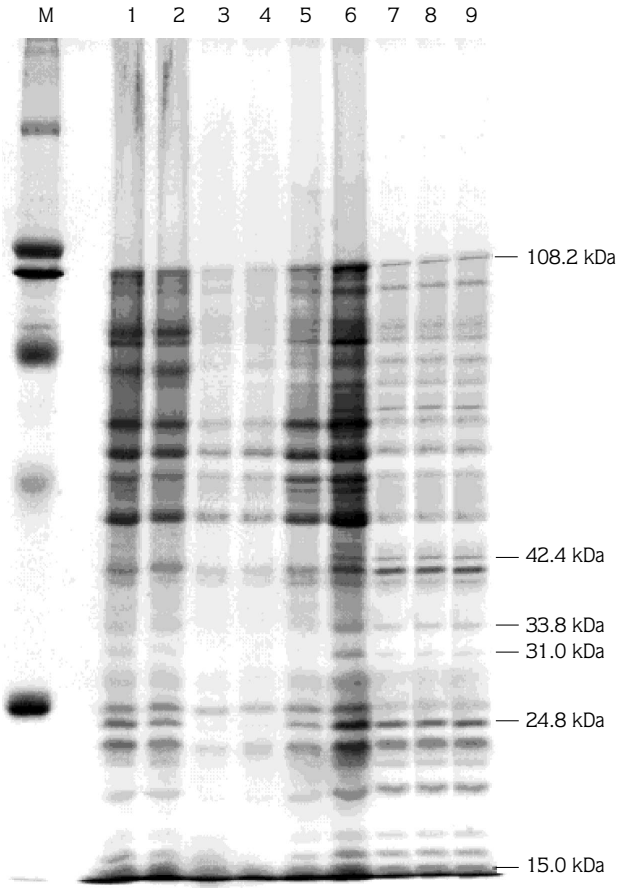

Figure 3. Protein profiles of *L. lactis* subsp. *cremoris* LC79 under normal and stress conditions: 1. High temperature, 30 min; 2. High temperature, 90 min; 3. low pH, 30 min; 4. Low pH, 90 min; 5. Osmotic stress, 30 min; 6. Osmotic stress, 90 min; 7. Low temperature, 30 min; 8. Low temperature, 90 min; 9. Wild-type, M: protein marker (205, 116, 97, 4, 66, 45, and 29 kDa).

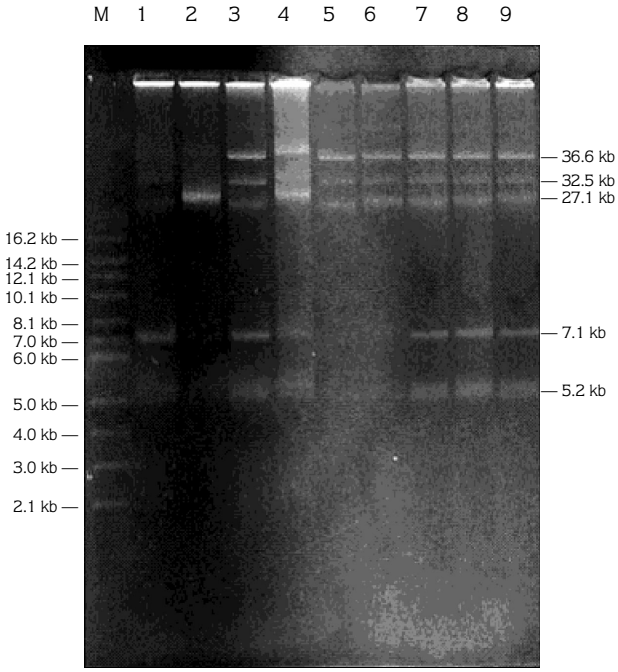

Figure 4. Plasmid profiles of *L. lactis* subsp. *cremoris* LC79 under normal and stress conditions: 1. High temperature, 30 min; 2. High temperature, 90 min; 3. Low pH, 30 min; 4. Low pH, 90 min; 5. Osmotic stress, 30 min; 6. Osmotic stress, 90 min; 7. Low temperature, 30 min; 8. Low temperature, 90 min; 9. Wild-type, M: plasmid marker.

industrial features, like lactose fermentation, proteolytic activity, production and resistance of bacteriocin, phage resistance, and fermentation of citrate, are encoded by plasmids (17,28,29). Thus, the stability under stress conditions of the plasmids in question is an essential characteristic, which helps to minimize the loss of time in industrial production.

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